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Resistance and Susceptibility of Mice to Bacterial Infection: Course of Listeriosis in Resistant or Susceptible Mice

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Resistance and susceptibility to *Listeria monocytogenes* in mice was found to be related to (i) the innate ability of the nonimmune macrophages to kill or inhibit the growth of the organism during the first 24 to 48 h after infection, and (ii) the time of onset of acquired cell-mediated resistance. Resistant C57Bl/6 mice were 10 times more efficient than susceptible BALB/c mice at suppressing the early growth of *Listeria* in the liver. Furthermore, the onset of acquired immunity occurred 24 to 48 h earlier in C57Bl/6 than in BALB/c mice. Acquired immunity was measured by (i) fall in bacterial numbers in spleen and livers of infected mice, (ii) adoptive transfer of immunity to normal mice by using spleen cells from infected mice, (iii) delayed-type hypersensitivity skin testing, and (iv) uptake of tritiated thymidine by lymphocytes in the spleen.

Listeria monocytogenes was chosen as an ideal organism to study genetic variation in cellular (rather than humoral) factors in immunity to bacteria. It provides an acute, potentially lethal infection in mice, and, furthermore, the basic mechanisms of immunity have been extensively studied both in vivo (16) and in vitro (3, 12). *Listeria* organisms are readily phagocytosed, but then survive and proliferate within macrophages. Acquisition of immunity depends on stimulation by bacterial antigens of T lymphocytes, which subsequently release lymphokines having the twofold effect of (i) increasing the bactericidal activity of macrophages, and (ii) attracting more bone marrow-derived monocytes to the site of infection. Antibodies are not able to transfer immunity to *Listeria* (11) and are not even detected in primary infection (17).

As the first part of this study, the accompanying paper (1) presents a study of the genetic basis of resistance and susceptibility to *L. monocytogenes* in mice. Evidence suggested that resistance was specified by one major dominant gene (or group of linked genes) that was not linked to any of a number of known genetic markers studied, including the recognized immune response genes linked to genes specifying *H-2* haplotype or immunoglobulin allotype. The possibility that the influence of the major gene could be modified by others was not ruled out.

To elucidate the mode of action of the *Listeria* resistance gene(s), we examined the role of variations in initial bactericidal activity of normal macrophages or in the various parameters of acquired cellular immunity. The strains of mice chosen were the resistant C57Bl/6 and susceptible BALB/c. The time course of bacterial numbers in the spleen and liver, where intravenously injected *Listeria* localize in mice, was studied in the two strains, together with a number of correlates of cell-mediated immunity. The latter included efficiency of adoptive transfer of immunity (9), delayed-type hypersensitivity (DTH) (2), and cell proliferation in the spleen (15).

It was found that the resistant and susceptible strains differed by two important parameters. These were the initial ability of the *Listeria* to proliferate in the normal animal and the time of onset of acquired cell-mediated resistance to infection.

MATERIALS AND METHODS

Mice. C57Bl/6 and BALB/c mice were bred in the Microbiology Animal Breeding Unit, Melbourne University, Australia, by strict brother-sister mating. Female mice only were used, between 8 and 10 weeks old.

Infection of mice. *L. monocytogenes* was injected intravenously (1).

Assay of infection. The spleens and livers of mice killed by cervical dislocation were homogenized individually in a tissue homogenizer (Measuring and Scientific Equipment Ltd., Sussex, England) in distilled water with 1% horse serum. Serial 10-fold dilutions were sampled in duplicate on nutrient agar plates. Colony counts were used to calculate the geometric mean of viable bacteria per organ.

Adoptive transfer of immunity. In experiments in which donor mice had been infected for at least 6 days, spleens from normal or infected mice were teased through 80-mesh stainless steel sieves into Eisen bal-

anced salt solution. Syngenetic recipients, which had been challenged 3 h earlier with about 10^4 *Listeria*, were injected intravenously, usually with a dose of 5 $\times$ 10^7 viable spleen cells. Injecting the bacteria before the cells ensured that the great majority were already within the host macrophages when the spleen cells were injected (15).

In experiments comparing the earliest times at which immunity could be transferred, it was necessary to use donor mice still bearing high numbers of *Listeria* in the spleen. Therefore, the cells were incubated overnight in Eagle minimum essential medium containing 10% fetal calf serum and 60 mg of penicillin and 100 mg of streptomycin per liter, by the method of Zinkernagel et al. (20). The cells were then washed three times with antibiotic-free Eagle medium and left on ice for 6 h in antibiotic-free medium to allow any remaining antibiotics to diffuse out. They were again washed, and 100 $\times$ 10^6 viable cells were injected intravenously into syngeneic recipients, already challenged with *Listeria* as described above. Viable bacterial counts failed to recover any *Listeria* from cells treated in this way, nor was there any evidence of carry-over of antibiotics depressing the number of *Listeria* in recipients of treated compared with untreated normal cells.

Protection of recipient mice was expressed as the difference between the logarithm of the number of bacteria in the spleen or liver or mice receiving normal or immune cells.

DTH. A protein extract of listeria antigens was prepared by ammonium sulfate precipitation of the supernatant of a Trypticase soy broth culture of *Listeria* (9). The precipitate was redissolved in phosphate-buffered saline (pH 7), dialyzed, concentrated through a Diaflo membrane (Amicon Corp., Lexington, Mass.), and separated into two fractions by passage through a Sephadex G-50 column. The higher-molecular-weight fraction was the active fraction. Protein concentrations were assayed by the Lowry method (5). Listeria antigen was injected intradermally in 0.03 ml into one hind footpad of each mouse, and foot swelling was measured 24 h later by Schnell-taster dial gauge calipers and by comparison with the uninjected feet (4). A swelling of 0.2 mm or more, which is readily palpable, was considered positive. The dose of antigen chosen was the highest that failed to produce positive reactions in normal nonimmune animals: 10 μg of protein per mouse.

Deoxyribonucleic acid (DNA) synthesis in spleen. Mice were injected intravenously with 10 μCi of tritiated thymidine ([^{3}H]TdR) (specific activity, 5 μCi/mmol) in 0.5 ml of saline (14). After 30 min the mice were killed and weighed samples of their spleens were homogenized in 5% trichloroacetic acid by using a pestle-type tissue homogenizer (Thomas Co., Philadelphia, Pa.). The precipitate was washed twice with 5% trichloroacetic acid and twice with ethanol. It was dissolved in 0.5 ml of Soluene 100 (Packard Instrument Co., Inc., Rockville, Md.) and then in 10 ml of scintillation fluid comprising 5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazolyl)benzene in 1 liter of toluene. Radioactivity was counted in a Packard automatic Tricarb liquid spectrometer.

RESULTS

Growth of *L. monocytogenes* in resistant or susceptible mice. C57Bl/6 (resistant) or BALB/c (susceptible) mice were injected intravenously with graded doses of *Listeria*. The doses were chosen on the basis of studies in the accompanying paper (1; Fig. 1) so that they were (i) sublethal for both strains; (ii) lethal for BALB/c, but sublethal for C57Bl/6; or (iii) lethal for both. The results are shown in Fig. 1, 2,

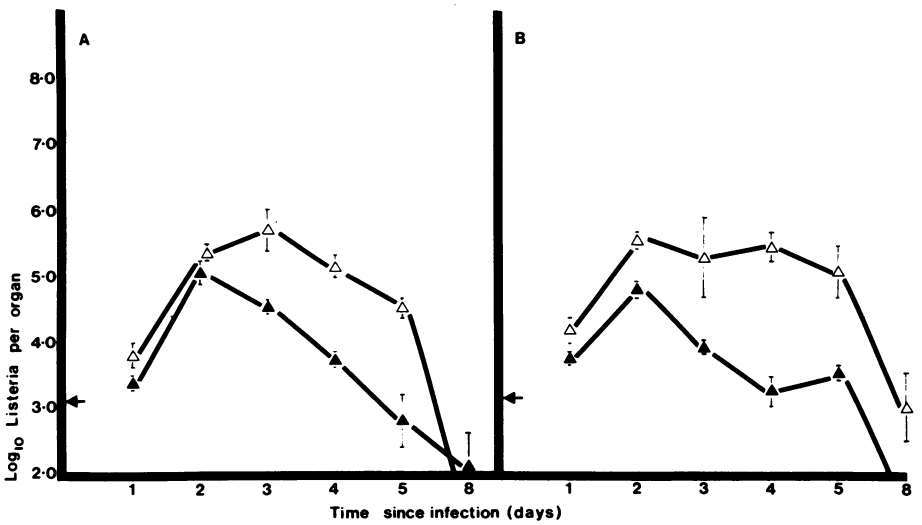

FIG. 1. *Growth curve of L. monocytogenes in spleen (A) and liver (B) of BALB/c (△) and C57Bl/6 (▲) mice infected intravenously with 1.2 $\times$ 10^3 viable organisms (arrow). Viable counts were performed on groups of five mice. Vertical bars, Standard errors.*

and 3, respectively, and show a marked difference between the two strains. (i) When a sublethal dose of 1.3×10^3 *Listeria* was given (Fig. 1), bacterial numbers in the spleens and livers of resistant C57Bl/6 mice increased until 2 days postinfection, then began to fall by day 3, and had virtually disappeared by day 8. By contrast, the number of *Listeria* in susceptible BALB/c mice given the same dose did not decrease until day 4 in the spleen and after day 5 in the liver. In the liver, the bacterial numbers in BALB/c mice even at the early time points were higher than in the C57Bl/6 mice. (ii) When the dose (2.2×10^4) was lethal for BALB/c, but not for C57Bl/6, there was again an early difference in bacterial numbers in the liver of mice of the two

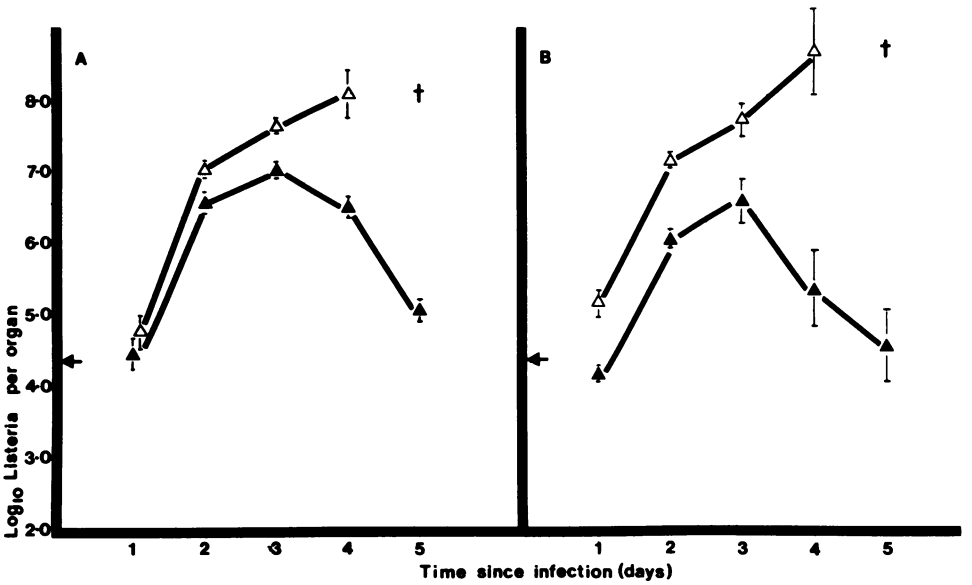

FIG. 2. *Growth curve of L. monocytogenes in spleen (A) and liver (B) of BALB/c (△) and C57Bl/6 (▲) mice infected intravenously with 2.2×10^4 viable organisms (arrow). Viable counts were performed on groups of five mice. Vertical bars, Standard errors. No BALB/c mice survived infection longer than day 4.*

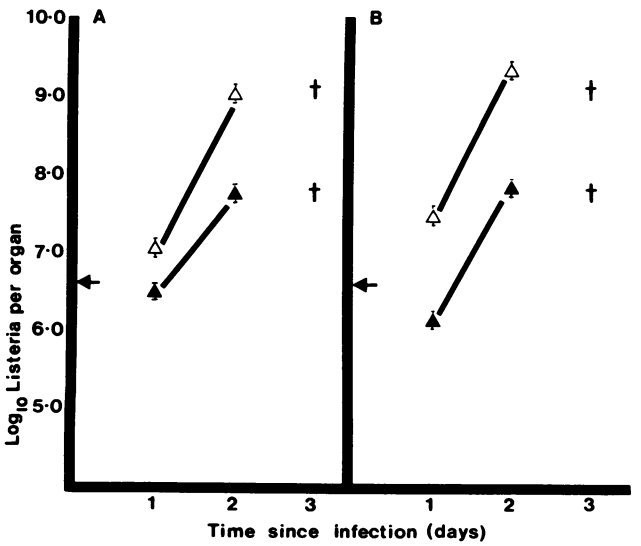

FIG. 3. *Growth curve of L. monocytogenes in spleen (A) and liver (B) of BALB/c (△) and C57Bl/6 (▲) mice infected intravenously with 4×10^6 viable organisms (arrow). Viable counts were performed on groups of five mice. Vertical bars, Standard errors. No mice survived infection longer than day 2.*

strains (Fig. 2). The fall in bacterial numbers in the spleen and liver or resistant C57Bl/6 mice was delayed until between days 3 and 4. However, by day 5 the number of organisms was markedly reduced. In the susceptible BALB/c mice, this reduction failed to occur, and all remaining mice were dead by day 5. (iii) When a lethal dose (4×10^6) was given, the lower bacterial numbers in resistant C57Bl/6 mice compared with susceptible BALB/c mice was still evident (Fig. 3), but all mice were dead by day 3. Thus, resistance in the C57Bl/6 mice was reflected in the lower numbers of bacteria as early as 24 or 48 h after infection and in the development of bactericidal activity earlier than that which occurred in BALB/c mice.

Early localization of *L. monocytogenes* in resistant or susceptible mice. Figures 1 to 3 show that even 24 h after infection, the resistant C57Bl/6 mice had some advantage over the BALB/c mice in having lower bacterial numbers in the liver and, less markedly, in the spleen. To check whether this might be due merely to differences in initial localization of the bacteria, mice were infected with a high dose of *Listeria* (8×10^5) and bacterial counts were performed on the spleen and liver 30 min, 3, 6, and 24 h later. Figure 4 shows identical numbers of organisms in the spleens and livers of resistant or susceptible mice up to 6 h postinfection, with 10-fold more in the liver in each case. By 24 h, however, it was evident that the resistant C57Bl/6 mice were able to retard bacterial growth, particularly in the liver, where the C57Bl/6 had only 1/10 the number of *Listeria* seen in BALB/c.

Acquired resistance to reinfection in resistant or susceptible mice. Groups of mice that had been given a sublethal (10^3) dose of *Listeria* 8 weeks earlier were challenged with graded doses of *Listeria* to determine their 50% lethal dose (LD_{50}). For comparison, the LD_{50} of *Listeria* in age-matched, previously uninfected mice was also checked. Table 1 confirms the differences in resistance of unprimed C57Bl/6 and BALB/c mice (1). In contrast, there was little difference in resistance of the two strains of secondary infection, showing that the susceptible BALB/c mice developed a satisfactory immunity. Indeed, in terms of increased resistance to secondary compared with primary infection, BALB/c mice show an almost 1,000-fold improvement, with C57Bl/6 mice showing a less than 10-fold improvement.

Adoptive transfer of immunity in resistant or susceptible mice. Mice were infected sublethally with approximately 10^3 *Listeria* organisms, and 7 days later, 5×10^7 viable spleen cells were used to adoptively transfer immunity to normal syngeneic mice that had been challenged 3 h earlier with 8×10^3 *Listeria*. The growth of *Listeria* in the spleens of these mice and in control mice that received normal cells was followed over the next 3 days. Although bacterial numbers were lower in the resistant C57Bl/6 mice than in the BALB/c mice, the log protection was the same in both (Fig. 5). The greatest protection was detected after 3 days, when it reached almost 4 logs, or 10,000-fold.

A comparison of the number of cells required to adoptively transfer immunity in the two strains was also made. Donor mice were infected

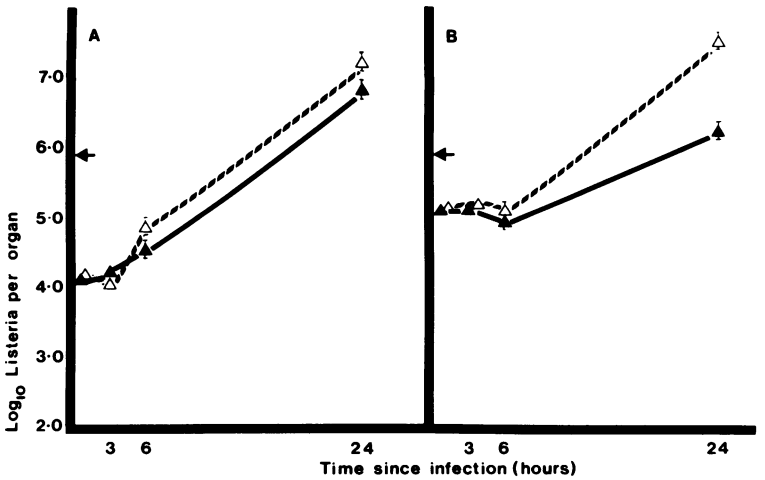

FIG. 4. *Early localization of L. monocytogenes in spleen (A) and liver (B) of BALB/c (△) and C57Bl/6 (▲) infected intravenously with 8×10^5 viable organisms (arrow). Viable counts were performed on groups of five mice. Vertical bars, Standard errors.*

with 10^3 *Listeria*, and 7 days later, 5×10^7, 2.5×10^7, 1.2×10^7, or 6×10^6 viable cells were used to adoptively transfer immunity. Bacterial numbers were assayed 2 days after adoptive transfer (Fig. 6). No significant difference was detected in the number of cells required to transfer immunity at this time after infection in resistant C57Bl/6 mice or in susceptible BALB/c mice. The protection conferred by 1.2×10^7 cells was equivocal, whereas 2.5×10^7 cells conferred 2 logs protection. Little advantage was gained by giving 5×10^7 cells at this time.

The time at which adoptive transfer became possible in the two strains was examined. Because spleens were to be obtained from mice still containing high bacterial numbers, it was necessary to resort to overnight incubation in antibiotic medium to prevent carry-over of *Listeria*, as described above. Bacteria were assayed 2 days after transfer. This procedure reduced the efficiency of adoptive transfer, but a valid comparison between the two strains could nevertheless be made. Figure 7A shows the time course of adoptive transfer in the two strains of mice, using donors infected with about 10^3 *Listeria* at different times before transfer. Significant protection was conferred by C57Bl/6 cells obtained 4 days after infection. By contrast, when BALB/c were the donors, significant protection did not appear until days 5 to 6.

TABLE 1. *Acquired resistance to lethal infection with Listeria in BALB/c and C57Bl/6 mice*[a]

Infection	BALB/c	C57Bl/6
Primary	2.5×10^3	8.7×10^5
Secondary	1.4×10^6	5.1×10^6

[a] LD_{50} of *Listeria* was compared in mice given a sublethal (10^3) dose of *Listeria* 8 weeks earlier, and in mice not previously infected.

DTH in resistant and susceptible mice. Mice infected with 10^3 *Listeria* were tested with listeria antigen injected into the footpads, and foot swelling was measured 24 h later. Figure 7B shows that a significant reaction occurred in resistant C57Bl/6 mice 3 days after infection (the antigen having been injected at 2 days), but a positive reaction was not seen in susceptible BALB/c mice until 5 days postinfection. That is, the response in BALB/c mice lagged 48 h behind that of C57Bl/6 mice. By 6 days postinfection, a strong reaction was seen in both strains. Hence, once again, the onset of cell-mediated immunity was earlier in C57Bl/6 than in BALB/c mice.

DNA synthesis in the spleens of resistant and susceptible mice. The uptake of [^{3}H]-TdR in the spleens of C57Bl/6 and BALB/c mice was followed during the course of infection (Fig. 7C). Once again, the resistant C57Bl/6 mice showed an earlier response than did the BALB/c mice, with a twofold increase in DNA synthesis by day 2, rising to a peak of 3.8-fold on day 4, and beginning to fall by day 5. The BALB/c mice showed only a slight response on day 3, but showed a 2.3-fold increase by day 4, and were still increasing their response on day 5.

DISCUSSION

The accompanying paper (1) described variations in the resistance of mice to *L. monocyto-*

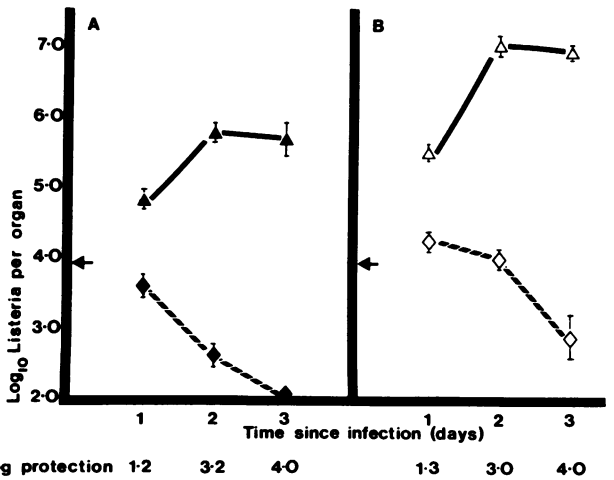

FIG. 5. *Syngenetic adoptive transfer of immunity to L. monocytogenes in the spleens of C57Bl/6 (A) and BALB/c (B) mice. Mice infected 7 days earlier with 10^3 Listeria were the donors of immune C57Bl/6 (◆) or immune BALB/c (◇) cells. Cells from uninfected C57Bl/6 (▲) or uninfected BALB/c (△) served as normal controls. 50×10^6 cells were transferred to syngenetic hosts that had been challenged 3 h earlier with 8×10^3 Listeria (arrow). Viable counts were performed on groups of five mice. Vertical bars, Standard errors.*

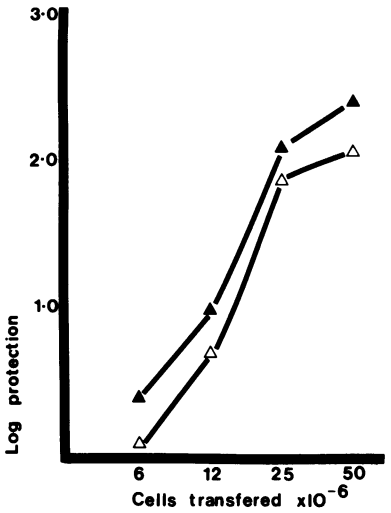

FIG. 6. *Number of cells required to adoptively transfer immunity to L. monocytogenes in the spleens of C57Bl/6 (▲) and BALB/c (△) mice. Mice infected 7 days earlier with 10^3 Listeria were donors of immune spleen cells, which were transferred in various numbers to groups of five syngeneic mice challenged 3 h earlier with 10^4 Listeria. Log protection was measured by comparison with mice of the same strain receiving 50×10^6 viable spleen cells from uninfected mice. Viable bacterial counts were performed 2 days after adoptive transfer.*

genes, apparently governed by a single dominant gene or group of linked genes. The present study sought to elucidate the important differences between genetically resistant C57Bl/6 and susceptible BALB/c mice. These differences might presumably lie in the innate ability of the nonimmune macrophages to handle the organism, or in variations in acquired cell-mediated immunity to this intracellular parasite. Differences between resistant and susceptible strains were, in fact, demonstrated in both natural and acquired immunity.

L. monocytogenes rapidly localizes in the macrophages of the liver and spleen of intravenously injected normal mice without the aid of opsonins (16). This initial localization of *Listeria* in the liver and spleen was identical in C57Bl/6 and BALB/c mice (Fig. 4). However, 24 h later, there was up to 10-fold more bacteria in the livers of BALB/c mice compared with those of C57Bl/6 mice, with lesser, but consistent, differences in the spleens. This difference was more evident at high, potentially lethal doses of *Listeria* (Fig. 2 and 3) than at lower doses (Fig. 1) and was maintained until acquired immunity was induced at 3 or 4 days. Apparently, the normal macrophages of the susceptible BALB/c mice are deficient, compared with C57Bl/6 macrophages, in their ability to kill or inhibit the

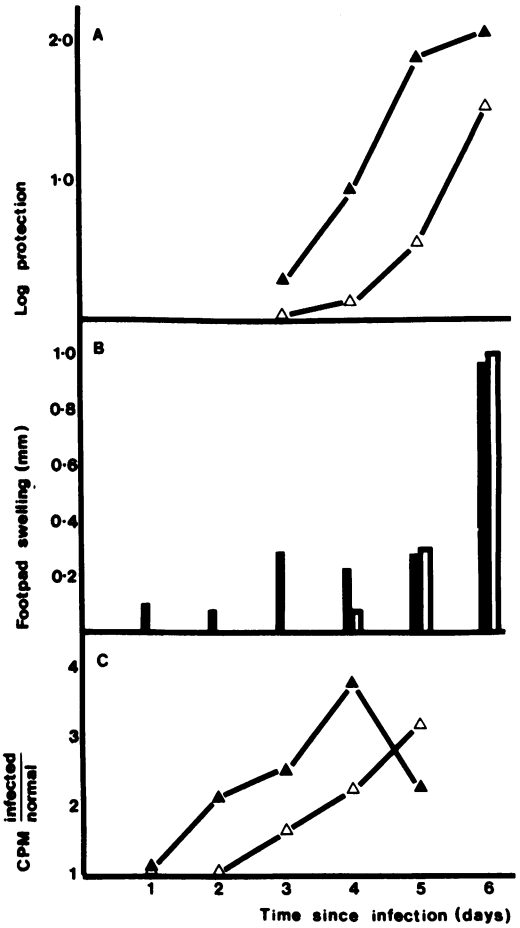

FIG. 7. *Time course during listeriosis of various parameters related to cell-mediated resistance to infection. (A) Time at which ability to adoptively transfer immunity is acquired. Mice were infected with approximately 10^3 Listeria, and at various times thereafter 50×10^6 viable spleen cells were transferred to syngeneic recipients. Normal controls were comprised of mice of the same strain given 50×10^6 cells from uninfected syngenetic mice. Log protection was measured as the difference in viable bacterial numbers 2 days after transfer in spleens of mice given normal or immune cells from C57Bl/6 (▲) or BALB/c (△) mice. (B) DTH in C57Bl/6 (solid bars) and BALB/c (open bars) mice. Groups of 5 mice were infected with 10^3 Listeria and tested for DTH at intervals. (C) DNA synthesis in spleens of C57Bl/6 (▲) and BALB/c (△) mice. Uptake of [^{3}H]TdR was measured at 24-h intervals in groups of five mice infected with 10^3 Listeria. The ratio of counts per minute (CPM) in infected compared with normal spleens was used as a measure of cell proliferation.*

growth of *Listeria* organisms. Whether this difference in normal macrophage bactericidal or bacteriostatic activity is a general property of the reticuloendothelial system handling of all

intracellular bacteria in these two strains or is specific for *Listeria* remains to be determined. It will be of particular interest to examine the early handling of *S. typhimurium*, since the resistance and susceptibility patterns of a variety of mouse strains are different for this organism and for *Listeria* (1, 10, 19).

The second critical difference between the two strains lies in the time of onset of cell-mediated immunity to the organism. This was measured in a number of ways, and by all parameters was found to occur 24 to 48 h earlier in the resistant C57Bl/6 mice.

The first and most direct way of measuring acquired immunity was the enumeration of bacterial numbers in spleens and livers to detect the onset of bactericidal activity. The numbers fell at least 24 h earlier in the C57Bl/6 mice than in the BALB/c mice, the precise time varying according to the infecting dose. For a sublethal dose of 10^3, the fall was evident by 3 days in spleens and livers of C57Bl/6 mice, whereas in BALB/c mice, splenic numbers did not increase until 4 days and in the livers there was a plateau and no significant fall until 5 days.

The second measure of acquired immunity used was syngeneic adoptive transfer by spleen cells. Judged by the disappearance of bacteria in the primary infection, immunity was well established in both strains by 7 days postinfection. When such 7-day infected mice were used as donors of immune cells for adoptive transfer, there was no significant difference in the efficiency of the two strains. Thus, when 5×10^7 normal or immune cells were transferred to normal recipients and the number of viable bacteria was assayed in the recipient spleens over the next 3 days, the log protection conferred by immune cells was virtually identical for the two strains (Fig. 5). No lag existed in the response of the macrophages in BALB/c recipients to the transferred cells, suggesting that once the T lymphocytes became activated, BALB/c macrophages were not deficient in their response to T cell-derived lymphokines. Furthermore, when the number of cells required to transfer immunity in the two strains was titrated 7 days after infection of the donor mice (Fig. 6), no significant difference was found. However, the time at which the ability to transfer immunity was acquired differed by 24 to 48 h (Fig. 7). Cells from C57Bl/6 mice were able to transfer significant protection 4 days after infection, whereas those from BALB/c mice were not effective until 5 or 6 days after sublethal infection. These times lagged a little behind onset of bactericidal activity in the primary infection, probably reflecting the relative inefficiency of adoptively transferred cells compared with cells in situ. However, the time difference was similar in each case.

Finally, the time course of two other correlates of cell-mediated immunity were examined. These were DTH, measured by the classical footpad swelling test (4), and synthesis of DNA in the spleen. DTH is believed to reflect similar mechanisms of T-cell activation, release of lymphokines, and influx of macrophages to that seen in cell-mediated resistance to infection (2). Furthermore, it is closely related chronologically to the sudden decrease in bacterial numbers with acquisition of immunity (8, 9). In the present experiments, positive reactions were seen 3 days after infection in C57Bl/6 mice, but not until 5 days after infection in BALB/c mice. Very strong DTH reactions were seen in both strains by day 6. Thus, DTH follows closely the fall of bacterial numbers in the two strains. On the other hand, increased synthesis of DNA in the spleen has been shown by North and colleagues (15, 18) to begin somewhat before the onset of acquired immunity, and this was the case in the present experiments. Uptake of [^{3}H]TdR in the spleens of C57Bl/6 mice reached double that in those of normal mice by 2 days postinfection, peaking by day 4. In the BALB/c mice, significant increases in [^{3}H]TdR uptake were achieved between days 3 and 4, and the rate was still rising at day 5 postinfection. North (15) has shown by autoradiography that [^{3}H]TdR administered in this manner is localized almost exclusively in proliferating lymphocytes in the spleen, although, in the liver, macrophages also proliferate (13). The relatively short labeling time minimizes the contribution of rapidly dividing cells recruited from the bone marrow. It may be noted that the peak of [^{3}H]TdR uptake occurs after the peak in bacterial numbers in the spleen (cf. Fig. 1 and 7), making it unlikely that bacterial DNA synthesis makes a significant contribution to [^{3}H]TdR uptake.

It is notable that once acquired immunity was established, BALB/c mice were in no way less efficient than C57Bl/6 mice at handling *Listeria* infection. When the resistance to lethal infection was tested in BALB/c and C57Bl/6 mice that had been sublethally infected 2 months earlier, the LD_{50} for both was in excess of 10^6 organisms (Table 1). The 2-month interval between primary and secondary infection ensured that no macrophages that had been activated by the primary infection remained (6, 17). Neither were the previously immunized BALB/c mice deficient in their ability to adoptively transfer immunity (Fig. 5 and 6).

Thus, we have shown differences both in the innate ability of macrophages from the two strains to kill or inhibit *L. monocytogenes* and in the time of onset of acquired cell-mediated

immunity. That these differences are quantitative rather than qualitative is in no way incompatible with their being governed by a single gene. In the extensively studied case of the immune response genes linked to the major histocompatibility complex, the strain difference is not between response and nonresponse, but between high and low response, which is further dependent on the antigen dose (9).

The question remains, however, of whether the effects on normal macrophages and on onset of specific immunity are related. Is the delay in acquired immunity related to differences in the T lymphocytes or to differences in the macrophages? There is no apparent lag in the response of BALB/c macrophages to immune T cells (Fig. 5). However, the macrophages could influence the response of the T lymphocytes in at least two ways. First, there may be differences in the presentation of antigen by macrophages to the T cells. Second, the overwhelming numbers of *Listeria* reached early in the infection of BALB/c mice through the apparent inability of the normal macrophages to control them could temporarily tolerize or paralyze the lymphocytes. Removal of viable bacteria during adoptive transfer by incubation of cells in antibiotic medium does not remove this putative paralysis. However, one argument in favor of this mechanism is the fact that when the dose of *Listeria* organisms given to C57Bl/6 mice was raised from 1×10^3 to 2×10^4, with an increase in the peak infecting numbers from 10^5 to almost 10^7, there was a delay in the onset of bactericidal activity from 3 to 4 days postinfection (Fig. 1 and 2). Furthermore, BALB/c mice given 2×10^4 *Listeria* were still showing rising bacterial numbers at day 4, whereas those given the lower doses were able to control their infection by that time.

To evaluate the relative role of T lymphocytes and macrophages, we are at present embarking on an investigation of the response of chimeric mice that combine cells from resistant and susceptible strains.

ACKNOWLEDGMENT

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